(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number
WO 01/25757 A1

(51) International Patent Classification[7]: G01N 21/65

(21) International Application Number: PCT/US00/27667

(22) International Filing Date: 6 October 2000 (06.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/157,958	6 October 1999 (06.10.1999)	US
60/168,936	3 December 1999 (03.12.1999)	US
60/169,339	6 December 1999 (06.12.1999)	US

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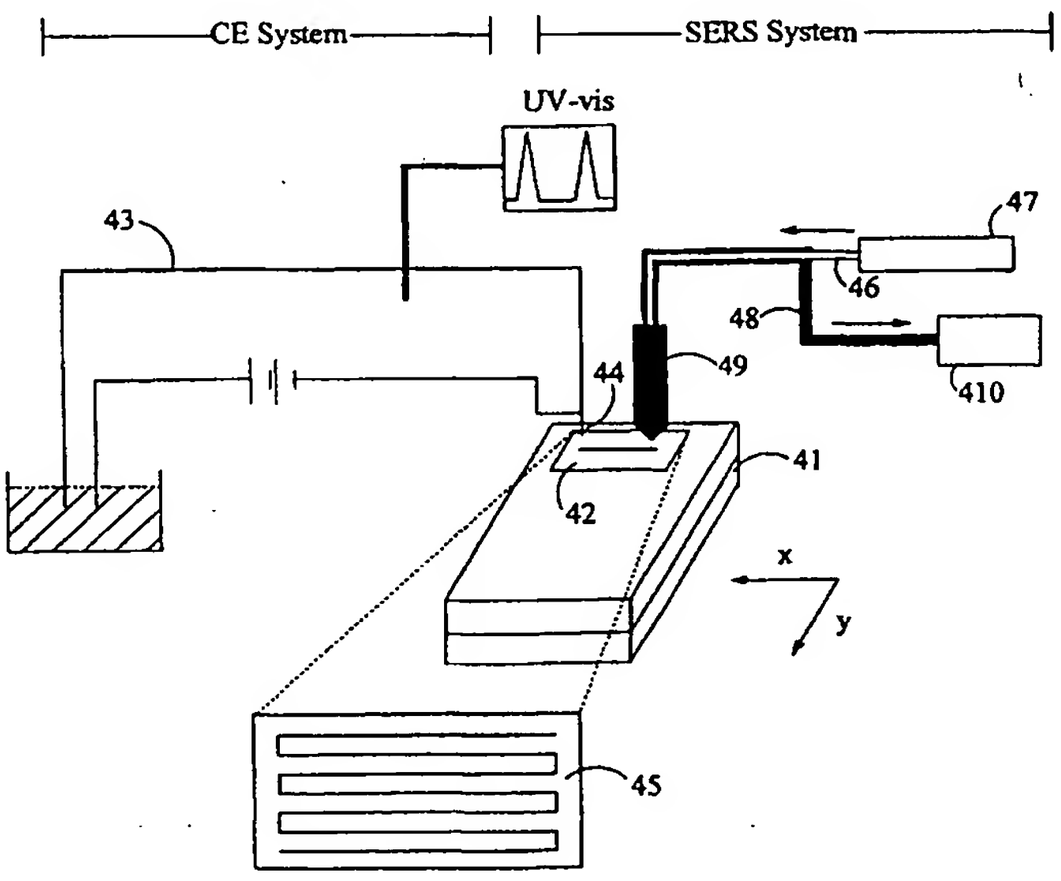
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NOVEL SURFACE ENHANCED RAMAN SCATTERING (SERS)-ACTIVE SUBSTRATES AND METHOD FOR INTERFACING RAMAN SPECTROSCOPY WITH CAPILLARY ELECTROPHORESIS (CE)



(57) Abstract: The invention provides novel Surface Enhanced Raman Scattering (SERS)-active substrates (42) for Raman spectroscopy that provide more SERS-enhancement and reproducibility than prior art substrates. The invention also provides a novel interface (45) between capillary electrophoresis (CE) and Raman spectroscopy.

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NOVEL SURFACE ENHANCED RAMAN SCATTERING (SERS)-ACTIVE SUBSTRATES AND METHOD FOR INTERFACING RAMAN SPECTROSCOPY WITH CAPILLARY ELECTROPHORESIS (CE)

Field of the Invention

The invention is directed to the technique of Raman spectroscopy, specifically to the technique of surface enhanced Raman scattering (SERS)-based Raman spectroscopy. The invention provides novel SERS-active substrates, and also provides a novel interface between SERS-based Raman spectroscopy and capillary electrophoresis (CE).

Background of the Invention

Raman spectroscopy is an ultrasensitive chemical analysis method, well known in the art. The technique relies on the Raman effect, in which the energy of photons that are incident on a molecule is coupled into distinct vibrational modes of the molecule's bonds. Such coupling causes some of the incident photons to be inelastically scattered by the molecule with a range of energies (wavelengths) that differ from the energy of the incident light. The energy difference between the incident photons (wavelength = $\lambda_{incident}$) and scattered photons (wavelength = $\lambda_{scattered}$) is termed the Raman shift. By calculating $1/\lambda_{incident} - 1/\lambda_{scattered}$, the Raman shift can be expressed numerically in wavenumbers (cm^{-1}).

A Raman spectrum is a plot of wavenumber versus intensity. Particular molecular structures deplete the incident photons of specific amounts of energy, thereby causing band(s) to appear at specific wavenumber positions in the Raman spectrum. The intensity value for each band is proportional to the concentration of the molecular structure. Hence, Raman spectroscopy can yield structural and quantitative data about samples of unknown molecular composition.

The incident illumination for Raman spectroscopy, usually provided by a laser, can be concentrated to a small spot if the spectroscope is built with the configuration of a microscope. Since the Raman signal scales linearly with laser power, light intensity at the sample can be very high in order to optimize sensitivity of the instrument. Moreover, because the Raman response of a molecule occurs essentially instantaneously (without any long-lived highly energetic intermediate states), photobleaching of the Raman-active

molecule by this high intensity light is impossible. This places Raman spectroscopy in stark contrast to fluorescence spectroscopy, where photobleaching dramatically limits many applications. In addition, Raman spectra can be acquired at any excitation frequency, thereby allowing an excitation wavelength to be chosen that minimizes adverse photochemical effects or background fluorescence.

The Raman effect can be enhanced at least 10^6 fold by bringing the Raman-active molecule(s) close to a structured noble metal surface (such as gold, silver, or copper), typically through absorption. Enhancement can also be observed when the Raman-active molecule(s) are brought close to structured surfaces of certain other metals as well (such as sodium and potassium). The mechanism by which this surface-enhanced Raman scattering (SERS) occurs is not well understood, but is thought to result from a combination of (i) surface plasmon resonances in the metal that enhance the local intensity of the light, and; (ii) formation and subsequent transitions of charge-transfer complexes between the metal surface and the Raman-active molecule. Substrates that demonstrate the SERS effect are referred to as SERS-active substrates, or SERS substrates. As used herein, the term SERS refers both to the physical phenomenon of surface enhanced Raman scattering, and also to Raman spectroscopy of analytes associated with SERS-active substrates.

Detection of submonolayers of Raman-active adsorbates can be easily achieved using SERS. Furthermore, SERS exhibits advantages in fluorescence discrimination, limited interference in aqueous media, and the ability to be used in a variety of sensing environments. SERS has also been successfully interfaced with a number of separation techniques, including gas chromatography, liquid chromatography, and flow injection analysis for the quantitative and qualitative analysis of polymers, dyes, environmental and biological molecules.

To date, SERS has been an invaluable analytical tool; however, the variability in signal response from SERS-active substrates limits the technique from attaining its enormous potential. It is known in the art that the optical properties of SERS-active substrates depend critically upon the feature size, shape, inter-feature spacing and the extent of coupling between surface features. The art contains reports of potential substrate architectures and preparation techniques. Examples include electrochemically-roughened electrodes, laser ablation of metals, aggregates of colloidal Au or Ag particles, chemically

etched metal surfaces, and evaporated metal films. In addition, an effective 2-layer SERS substrate comprising a microarray of Ag-coated colloidal Au immobilized on a silanized glass surface is described in Bright, R. M *et al*, "Chemical and Electrochemical Ag Deposition onto Preformed Au Colloid Monolayers: Approaches to Uniformly-sized Surface features with Ag-Like Optical Properties" *Langmuir* 12:810-817 (1996), incorporated herein by reference in its entirety. This 2-layer SERS substrate combines the favorable properties of Au – namely monodispersity and ease of manipulation – with the superior SERS enhancement factors of Ag.

Among these prior art techniques, the preparation of SERS-active substrates with vapor deposited Ag films is popular due to its stability and handling ease. The dependence of SERS activity on the morphological and optical properties of vapor-deposited thin metal films has been extensively studied in the art as a function of deposition rate, geometry, and temperature. To better control surface roughness, several attempts have also been reported by immobilizing microparticles such as latex beads, fumed silica, and alumina powder on the surface before film evaporation. While the substrate enhancement has been improved to a certain level, the reproducibility of substrates preparation still remains a important limiting factor in many SERS applications. It is an object of the present invention to provide new SERS-active substrates that address these prior art limitations. Specifically, it is an object of the invention to provide SERS substrates that have increased enhancement factors and production reproducibility.

It is also an object of the present invention to expand the application of SERS as a detection method. In particular, it is an object of the invention to interface SERS with the technique of capillary electrophoresis (CE). CE is known in the art for its rapid, efficient, high-resolution analysis of nanoliter-volume samples, and has become a powerful separation technique used for a variety of analytes ranging from simple metal ions, to large molecules like proteins and DNA fragments. CE is typically performed by injecting a sample at one end of a long fused silica capillary (in some applications up to 100 cm in length with an inner diameter of less than 10 μm), then applying a very high potential difference across the two ends of the capillary. Analytes within the injected sample are separated from one another based on its charge/size ratio in the capillary eluant at different times. The scope of this technique has been further expanded with the emergence of

capillary array electrophoresis, a major technology used for the Human Genome Project, and miniaturized CE, where biological assays can be performed on a single chip.

Despite poor sensitivity, on-column UV absorbance detection is, by far, the most common means of monitoring CE separation due to its low cost and flexibility. Laser-induced fluorescence (LIF) offers much greater sensitivity, but requires fluorescent analytes. While derivatization strategies have been developed to enable detection of otherwise non-fluorescent analytes, these processes can be time-consuming and difficult. Other detection approaches, including electrochemical, thermo-optical, and chemiluminescence methods have been explored with varying degrees of success. However, none of the aforementioned techniques can provide qualitative information about the analytes beyond retention times. Since structure determination by retention time alone requires extensive knowledge of the sample beforehand (*i.e.*, a knowledge of the likely components of the sample, coupled with knowledge of the retention time of each component under the particular experimental conditions used), the identification of unknown compounds using these methods is quite complicated.

To circumvent this drawback, mass spectrometry (MS) has been developed as an alternate detection method for CE. MS provides molecular weight data and structural information based on fragmentation patterns, and has been successfully interfaced with CE. However, this technique often requires a complicated CE/MS interface, which poses a challenge in instrumental design.

Morris and coworkers have demonstrated online conventional (non-SERS) Raman detection for CE. For example, they have reported detection of 10 μM nitrate (620 ppb) and perchlorate (1 ppm), in which Raman bands of nitrate (1047 cm^{-1}) and perchlorate (934 cm^{-1}) were measured in a separation time of less than 3 minutes. Unfortunately, the inherently weak signal of Raman scattering makes detection of submicromolar concentrations extremely difficult. The detection limit is typically in the range of ~10^{-7} M with the aid of sample preconcentration via isotachophoresis.

Nirode and coworkers have demonstrated direct on-column SERS detection in CE using colloidal Ag in the running buffer as the SERS substrate, as described in *Anal. Chem.* 72:1866-1871 (2000), incorporated herein by reference in its entirety. In this technique, a Raman microprobe instrument was used to obtain Raman spectra through the wall of the capillary during electrophoresis ("on-the-fly" SERS detection). The detection

limit using this approach was found to be approximately 10^{-9} M for the model analyte studied (Rhodamine 6G). Despite the apparent promise of this method, there are numerous drawbacks. First, the presence of colloidal Ag in the running buffer can be expected to interfere with the electrophoretic separation of some samples. Second, colloidal Ag is a rather crude, variable, and non-optimal SERS substrate, so the full sensitivity potential of SERS cannot be realized. Third, only a small portion of the analyte appears to be bound to the colloidal Ag during CE. Fourth, to minimize artifacts caused by performing SERS on a moving analyte, the electrophoretic voltage must be kept low (thus extending the time of the electrophoretic separation), and short SERS spectra integration times must be used (leading to low sensitivity). Finally, Ag particles gradually build up on the walls of the capillary, leading to a degradation in the SERS response of the analytes.

It is an object of the invention to provide an interface between CE and SERS that allows SERS substrates with the highest enhancement factors to be used, including the novel SERS substrates provided by this invention.

Summary of the Invention

The invention provides novel SERS substrates. In preferred embodiments, the substrate comprises a submonolayer of microparticles on a solid support, each microparticle comprising a Ag-clad colloidal Au particle, over which microparticles lies a thin, non-continuous layer of Ag film. The 3 layer substrate displays excellent reproducibility: spectral deviations within different regions of a single 3 layer substrate are of the same magnitude as spectral deviations observed among individual 3 layer substrates from different manufacturing batches. In addition, the 3-layer SERS substrate allows the routine detection of analytes in sub-picogram amounts.

The invention also provides a novel interface between capillary electrophoresis and SERS that allows the use of the 3-layer SERS substrate for eluant detection. In preferred embodiments, eluant leaving the capillary is deposited onto a moving SERS substrate to form a linear eluant trail. The eluant trail is then analyzed by a Raman microscope, either immediately after each eluant drop is deposited, or after the entire eluant trail is deposited. The eluant trail preserves in a spatial format the temporal separation achieved by CE. Consequently, separation results can be preserved for later examination or for further

analysis using a second detection method. Because the electrophoresis and SERS steps are performed separately, the two steps can be independently optimized, allowing the full sensitivity of SERS to be exploited. Furthermore, "offline" SERS detection allows the full length of the capillary to be used for separation, thereby maximizing the resolution capability of CE.

Brief Description of the Drawings

Figure 1 depicts schematically stages in the synthesis of Au-Ag-Ag 3-layer SERS-active substrates.

Figure 2 illustrates the enhancement in Raman intensity provided by an Au-Ag-Ag 3-layer SERS-active substrate.

Figure 3 shows the detection sensitivity of 1,3,5-Triazine using an Au-Ag-Ag 3-layer SERS-active substrate.

Figure 4 depicts schematically the interface between SERS-based Raman spectroscopy and capillary electrophoresis.

Figure 5 illustrates the detection by Raman spectroscopy of *trans*- 1,2-bis(4-pyridyl) ethylene (BPE) deposited onto a SERS-active substrate by capillary electrophoresis.

Figure 6 shows the time course for separation of BPE and N,N-dimethyl-4-nitrosaniline (p-NDMA) by capillary electrophoresis as monitored by UV-visible spectroscopy (FIGURE 6A) and SERS (FIGURE 6B).

Figure 7 shows full Raman spectra for each of points a-f identified in FIGURE 6B.

Figure 8 shows a time course for separation of BPE and p-NDMA by capillary electrophoresis as monitored by Raman spectroscopy at wavenumbers where only BPE (curve 2) or p-NDMA (curve 3) show a Raman response.

Figure 9 shows a UV-vis spectrograph (FIGURE 9A) and Raman spectra (FIGURES 9B and 9C) for the separation by capillary electrophoresis of a mixture of tyrosine and tryptophan.

Figure 10 shows a UV-vis spectrograph (FIGURE 10A) and Raman spectra (FIGURES 10B and 10C) for the separation by capillary electrophoresis of a mixture of chlorophenol (CP) and dichlorophenol (DCP).

Detailed Description of the Preferred Embodiments

3-Layer SERS Substrates

In one embodiment, the invention provides a 3 layer SERS-active substrate comprising a submonolayer of microparticles attached to a solid support, each microparticle comprising a colloidal particle of first noble metal clad with a second noble metal, and said microparticles overlaid with a thin, non-continuous film of said second noble metal.

In preferred embodiments, the substrate can be synthesized by first immobilizing the colloidal metal particles comprising the first noble metal on a solid support, then coating the immobilized colloidal particles with a plating solution containing the second noble metal in a chemically reduced form. Finally, the resulting clad colloidal particles are evaporatively coated with a discontinuous layer of the second noble metal.

In preferred embodiments, the first noble metal is Au and the second noble metal is Ag. SERS substrates of this composition are referred to as Au-Ag-Ag substrates. Preferably, the Au colloid is approximately a 12 nm diameter colloid, and the Ag film is 10 nm – 30 nm in thickness, most preferably 20 nm in thickness.

FIGURE 1 illustrates schematically the stages in the one embodiment of synthesis of Au-Ag-Ag substrates. A glass slide 10 is first derivatized with an organosilane, such as 3-mercaptopropylmethyl-dimethoxysilane (MPMDMS). MPMDMS forms a self-assembled monolayer 11 on the glass surface; Au colloid 12 can bind via the thiol group of MPMDMS to form a Au colloid microarray on the glass slide. Other possible methods and reagents for immobilizing Au colloid on a glass slide are known to those skilled in the art. Following immobilization, the Au colloid is then treated with chemically reduced Ag^+ from a plating solution, such as, for example, LI Ag plating solution (available from Nanoprobes, Inc.) to form a Ag coating 13 over the Au colloid. Finally, a discontinuous layer of Ag 14 is evaporated over the Ag-clad Au colloid by placing the glass slide in thermal evaporation system. Example 1 provides an example of a detailed protocol for the synthesis of Au-Ag-Ag substrates.

It is known in the art that the optical properties of SERS-active substrates depend critically upon the feature size, shape, inter-feature spacing, and the extent of coupling between the surface features. In preferred embodiments, the colloid size, the rate of evaporative deposition, and the length of the evaporative deposition step (thickness of the

overlayer of the second noble metal), are varied in order to maximize the SERS enhancement of the 3-layer substrate. In especially preferred embodiments, these factors are chosen so as to obtain a substrate in which the evaporated second noble metal both forms discrete "islands" between the colloid particles, and also enlarges the size of the colloid particles. Without being bound by a single theory or hypothesis, it is believed that this substrate morphology enhances SERS activity by both increasing the surface area for analyte adsorption, and also by increasing the electromagnetic enhancement through the "rod-like" effect, as described in Creighton, J. A. *The Selection Rules for Surface-Enhanced Raman Spectroscopy*, Clark, R. J. H. and Hester, R. E., Ed.; John Wiley & Sons Ltd., 1988; Vol. 16, pp 37-89, incorporated herein by reference in its entirety. For Au-Ag-Ag substrates using a 12 nm colloid, preferably a 20 nm thick overlayer of Ag is evaporatively deposited at a rate of 0.1 nm s^{-1} – 0.5 nm s^{-1}. Images of SERS substrates can be obtained either by Atomic Force Microscopy (AFM) in tapping mode, or by Field Emission Scanning Electron Microscopy (FE-SEM).

FIGURE 2 illustrates the enhancement in the SERS activity of the 3-layer substrates provided by the invention by comparing the SERS spectra of 2 μL of 0.1-mM *trans*- 1,2-bis(4-pyridyl) ethylene (BPE) solution drop-coated onto each of the substrates illustrated in FIGURE 1. No SERS signal was observed on either the merely silanized glass surface (trace a) or the colloidal Au microarray surface (trace b). A pronounced SERS signal could be observed on the 2-layer substrate (Ag-clad Au colloid) (curve c). A 3 layer Au-Ag-Ag substrate (20 nm thick Ag overlayer) (curve d) has an approximately 20% decrease of fluorescence background and a more than 3-fold signal enhancement relative to the 2-layer substrate (measured by integrating peak area from 1071 cm^{-1} to 1271 cm^{-1}). Example 2 gives experimental protocols used to obtain the data presented in FIGURE 2.

The 3-layer substrates of the instant invention are useful in any application where it is necessary to detect the presence of a particular analyte(s). Because of their large enhancement factor, the present substrates are especially useful for the detection of analytes that are present at low concentrations. In particular, the substrates are useful for the sensing of biomolecules that are present at low concentrations in biological fluids. The sensitivity of the 3-layer SERS substrates of the instant invention is demonstrated in FIGURE 3 for the environmentally important compound 1,3,5-Triazine. 1,3,5-Triazine is

the parent compound of the most prevalently used herbicide family. FIGURE 3 illustrates a plot of the logarithmic concentration value of 1,3,5-Triazine against the normalized peak area of the 926 cm^{-1} band (from ring breathing) that is characteristic of the compound (using 12 mW of 633 nm excitation illumination). The peak area has been normalized by taking the quotient of the raw peak area and integration time. The inset to FIGURE 3 shows the SERS spectra of 2 μL of 8 x 10^{-3} M (30 second integration) and 8 x 10^{-8} M solution (45 seconds) of 1,3,5-Triazine. It can be seen that the 3-layer substrates of the instant invention allow for the detection of picogram quantities of 1,3,5-Triazine, at low excitation powers and in less than a minute of integration time.

The 3-layer SERS substrates of the instant invention are a significant improvement over prior art SERS substrates not only because of their increased SERS activity, but also because of the reproducibility with which they can be fabricated. Table 1 compares the peak areas for the 1010, 1200, and 1610-1640 cm^{-1} SERS bands of 10 mM BPE taken at five different spots on the same 3-layer substrate. Good reproducibility between spectra is observed, with all relative average deviations less than 13 %. Similar reproducibility can be realized for substrates prepared simultaneously and in separate substrate batches; a substrate batch is defined as a group of slides, typically eight due to limitations of evaporation chamber space, who simultaneously undergo each fabrication step. Table 2 gives peak area comparisons for SERS spectra of 10 mM BPE on substrates from six different substrate batches. Again, good reproducibility is observed, and the average relative deviations is of the same magnitude as a single substrate, with all values less than 12 %. It should also be noted that the data was collected for batches fabricated over an eight-month period, further demonstrating the ability to make reproducible substrates of the instant invention.

Interfacing Capillary Electrophoresis with SERS

In another aspect of the invention, a method is taught to interface SERS substrates with capillary electrophoresis (CE). In preferred embodiments, a SERS substrate is placed underneath the outlet of an electrophoresis capillary such that eluant from the capillary -- containing resolved analytes – is deposited onto the SERS substrate. The SERS substrate is associated with translation means to enable the resolved analytes with the eluant to be deposited at different regions of the SERS substrate. FIGURE 4 illustrates an especially

preferred embodiment in which the translation means is a computer controlled x-y translation stage 41, wherein the computer controls the position and velocity of substrate 42. The substrate can be translated in such a way that the eluant leaving capillary 43 at outlet 44 is deposited in a linear trail – such as a continuous "S" pattern 45 – that preserves the temporal separation achieved by CE. In order to improve the visibility of the eluant trail, a marker dye may be included in the eluant. One suitable dye contemplated by the invention is Kiton Red 620.

Any translation pattern that deposits the eluant in a linear, non-overlapping fashion is contemplated by the invention. In addition, any SERS substrate that can be used to deposit a linear eluant trail is also contemplated. For example, eluant can be deposited on a cylindrical SERS substrate as a ring trail or spiral trail. Eluant can be deposited either continuously, or dropwise.

In one embodiment, following deposition of the eluant trail, Raman microscopy is used to acquire a Raman spectrum at each point along the trail. In preferred embodiments, a Raman spectroscope with a remote fiber-optic probe (often referred to as a Raman microprobe) is employed to retrace the eluant trail. Such an instrument is depicted in FIGURE 4. Laser excitation light 46, preferably from a He-Ne laser 47 (632.8 nm), is introduced by the optical fiber 48 into the back focal plane of a microscope objective 49, then focused onto the SERS substrate 42. SERS light from the substrate is then collected by the microscope objective 49, and directed to the Raman spectrometer 410 by the optical fiber 48. Preferably, the microscope objective has a focal length of between 3 mm – 8mm, leading to a laser spot size of 5 μm – 10 μm on the SERS substrate. Most preferably, a 3 mm objective is used to focus 20 mW of incident illumination, allowing a SERS spectrum to be acquired in < 5s. For longer acquisition times, it is preferable to use a larger spot size. It is routine experimentation for those skilled in the art to determine an appropriate spot size, laser power, and integration time for particular applications of the methods of the invention. Those skilled in the art will realize that the use of higher laser fluxes may cause sample damage at long exposure times, but will give an increased SERS signal relative to lower laser fluxes.

FIGURE 5 illustrates data obtained from one embodiment of the invention in which a 10 mM BPE solution was subjected to CE, then deposited onto (a) a 2-layer SERS substrate (Ag-clad Au colloid monolayer) and (b) a blank glass slide, and then examined

by Raman microscopy (3 mm objective, 20 mW of 632.8 nm illumination, 5 second integration). The complete lack of Raman response obtained from the BPE on the blank glass slide provides ample evidence that conventional Raman fails as a detection method for these low levels of analyte. In contrast, the response observed from the Ag-clad Au colloid monolayer substrate clearly demonstrates that SERS is a viable alternative to conventional Raman for identification of analytes separated by CE.

In prior art UV-visible spectroscopic detection or fluorescence detection of CE eluants, an initial run of each analyte in purified form is necessary to obtain individual retention times. The analyte in the mixture can only be identified by comparing the retention time with previously established standards, based on the assumption that the presence of other analytes in the mixture will not affect the individual migration velocity. Furthermore, if the eluant contains analytes that were not previously run as standards, then it can be extremely difficult to determine the composition of the eluant. Hence, the prior art methods are useful only when (i) the likely analyte composition of the eluant is known; and (ii) purified quantities of the individual analytes are available. In the SERS eluant-retracing method provided herein, however, a full Raman spectrum is recorded for every point along the eluant path. Thus, a single trace over the eluant-deposited substrate is sufficient to determine analyte identity based solely on the characteristic Raman spectrum of each analyte. Therefore, it is not necessary to determine the retention time beforehand of each analyte to be detected in the eluant. Indeed, unlike prior art detection methods, it is often not necessary in the SERS-retracing method to have prior knowledge of the identities of the analytes that the eluant contains. This means that the methods provided herein can be used to determine the composition of eluants without any prior knowledge of the likely composition of the eluant.

Analyte identification based on the SERS-retracing method provided herein is far more accurate than prior art analyte identification techniques that are based on retention time alone. Because the Raman spectrum of each analyte is complex (often consisting of ten or more discrete peaks), it provides an unambiguous and unique "fingerprint" for that analyte. By contrast, prior art UV-vis and fluorescence spectra provide just a single peak that cannot alone identify an analyte without knowledge of retention times.

The SERS-retracing method provided herein obviates the need to use analyte retention time as the exclusive basis for analyte identification. However, it is important to

note that the eluant trail preserves, in a spatial format, the temporal resolution achieved by CE. Specifically, each position on the SERS substrate represents a different elution/retention time point. In some embodiments, rather than retracing the entire eluant trail the Raman spectroscope can examine just those timepoints where the analyte of interest would be expected to be eluted based on prior knowledge of retention time. In other embodiments, the eluant trail can be examined first by Raman spectroscopy, and then specific time points on the substrate can be examined by other detection methods, such as mass spectrometry. This is a significant advantage over prior art attempts to interface CE with SERS using colloidal Ag in the running buffer as the SERS-active substrate. In summary, the creation of an eluant trail on a SERS substrate provides an extremely flexible, accessible, and data-rich source for eluant analysis.

Using prior art UV-vis and fluorescence detection techniques, a substantial amount of effort is often needed to reach a base-line separation in order to draw quantitative results for mixtures of analytes. In some instances, different analytes may have similar capillary migration times, causing them to be poorly resolved from one another. As a result, the UV-vis or fluorescence peaks of such analytes partially or substantially overlap, making quantitation difficult or impossible. Using the SERS-retracing method provided herein, it is possible to perform quantitative analysis of analyte mixtures by monitoring the intensity changes at the wavenumber range where only one analyte shows a Raman response. As a result, less effort is needed to achieve base-line separation, and even poorly resolved analytes can be accurately quantitated.

Unlike prior art attempts to interface SERS with CE by using colloidal Ag as the SERS-active substrate, the SERS-retracing method of the instant invention allows the use of optimized SERS-active substrates. In some embodiments, the SERS substrate is the 2-layer substrate comprising Ag-clad Au colloid. In preferred embodiments, the SERS-retracing method is used with the 3-layer substrates provided by the instant invention, most preferably with Au-Ag-Ag 3-layer substrates.

The utility of the methods provided herein are demonstrated by the data presented in FIGURES 6-8, illustrating SERS detection of CE-separated BPE and N,N-dimethyl-4-nitrosaniline (p-NDMA). As a point of reference for the effectiveness of the SERS-retracing method, UV-vis analysis of the separation is presented (FIGURE 6A). The UV-vis elution profile shows two distinct but not fully separated peaks. In SERS detection, the

constant speed of the translation stage allows the spatial location of both BPE and p-NDMA deposited on the substrate to be converted into a time scale to obtain information on the analyte retention times. FIGURE 6B shows the time course of the changes of integrated SERS intensity in the wavenumber range 1600-1650 cm^{-1}, a region where both BPE and p-NDMA display Raman response. Inspection of the retention times for both eluants shows agreement with those recorded by UV-vis, illustrating that the CE-SERS interface can perform the same detection function as UV-vis.

FIGURE 7 shows SERS spectra corresponding to each of the points indicated in the lower panel of FIGURE 6. Spectra a, e and f represent three examples of SERS spectra of the background, a pH 6.8 phosphate buffer with a low SERS response from 1000 cm^{-1} to 1700 cm^{-1}, that were taken along the CE-deposited eluent trace. The only Raman activity observed for these positions were two sizable peaks at 616 cm^{-1} and 920 cm^{-1}, which are attributed to components in the LI Ag plating solutions and were present in all SERS spectra collected; their intensities were markedly decreased in the presence of analyte, presumably due to competition for surface sites (data not shown). Spectrum b was taken at a position (t=946 s) corresponding to BPE eluant. As expected, distinct BPE characteristics are observed (e.g., bands at 1200 cm^{-1} from C=C stretch and 1010 cm^{-1} from ring breathing)." Spectrum c, recorded at a position corresponding to t=949 s, where the eluants were incompletely separated, exhibits SERS characteristics of both analytes. Spectrum d (t=952 s) shows spectral features corresponding to p-NDMA, including a 1166 cm^{-1} band from phenyinitroso stretch on Ag-coated surfaces, once again confirming the utility of the SERS-retracing method provided herein as a structure-specific detection method for CE separation.

FIGURE 8 shows a time course of integrated SERS intensity from 1230-1180cm^{-1} (curve 2), the range in which only BPE showed Raman response, and a plot of SERS intensity from 1180-1130 cm^{-1} (curve 3) which is unique for p-NDMA response in this experiment. Curve 1 is the base line curve showing SERS intensity at 1600-1650 cm^{-1} where both BPE and p-NDMA show a Raman response. This data demonstrates how the methods of the instant invention enable quantitative analysis of poorly-resolved analyte mixtures by monitoring the intensity changes at the wavenumber range where only one analyte shows a Raman response.

The methods of the instant invention are especially well suited to biological assay applications, where analytes are typically present at low concentrations. As an example of such an application, FIGURE 9 illustrates SERS data from a CE separation of the amino acids tyrosine and trytophan (1:1 ratio). FIGURE 9A shows the UV eluant profile for the separation, and reveals a significant interval in retention times (several minutes between peaks). FIGURE 9A and FIGURE 9B show the corresponding SERS spectra of tyrosine and tryptophan, respectively. For example, the peak at 837 cm^{-1} (FIGURE 9B) is characteristic of the ring breathing mode of the p-hydroxyphenyl moiety in tyrosine, while the 1334 cm^{-1} Raman band (FIGURE 9C) derives from the indole ring of tryptophan . A number of other bands further served to identify, both analytes, namely those that appear at 1176 and 1603 cm^{-1} for Tyr and at 1012 and 1607 cm^{-1} for Trp, all of which correspond well to the Raman spectra of the respective amino acids. The data in FIGURE 9 was obtained using the methods of Example 3 and 1 mg/ml solution of tyrosine and trytophan.

The methods of the instant invention are also well suited to the separation and identification of environmental pollutants. As an illustration of this role, FIGURE 10 shows data from the application of the SERS-retracing method to a mixture of chlorophenols. FIGURE 10A shows the UV eluant profile of 2-chlorophenol (CP) and 2,4-dichlorophenol (DCP). SERS spectra for the eluted CP and DCP are presented in FIGURE 10B and FIGURE 10C, respectively. Specific bands observed from the SERS spectra can be used to identify each of the compounds. The 562 cm^{-1} band in FIGURE 10B is a strong indication of the presence of ortho-disubstituted benzene, while the peaks at 851, 1143 and 1285 cm^{-1} in FIGURE 10C correspond well with the Raman spectrum of pure dichlorophenol. Note that the Raman bands of DCP at 700 and 1028 cm^{-1} overlapped bands from propanethiol, a compound used to coat the substrate in order to improve the adsorption of hydrophobic compounds.

Examples

Example 1

Preparation of 3-Layer Substrates

Materials Glass slides used were Fisher Premium Microscope Slides. Water used for all experiments was 18.2 MΩ, distilled through a Barnstead nanopure system. 3-

mercaptopropyl methyldimethoxysilane (MPMDMS) was obtained from United Chemical. $HAuCl_4 \cdot 3H_2O$, $AgNO_3$, trisodium citrate dihydrate, NaOH were obtained from Aldrich. 12-nm colloidal Au was prepared from $HAuCl_4 \cdot 3H_2O$ reduced by citrate, as described in Grabar, K. C. *et al, Anal. Chem.* 67:735-743 (1995), incorporated herein by reference in its entirety. All references to "12-nm Au" particles in this text were nearly spherical, with a standard deviation of less than 2 nm. Particle size was determined by transmission electron micrographs for each preparation using NIH Image software (available over the internet at www.nih.gov). LI Ag solution was purchased from Nanoprobes and used as directed. Concentrated HCl, HNO_3, H_2SO_4 and 30% H_2O_2 were purchased from J. T. Baker Inc. Spectrophotometric grade CH_3OH was obtained from EM science. Ag (99.99%) shot used for evaporation was obtained from Johnson-Matthey Corp.

Substrate Preparation Glass slides were cut and cleaned as described in Grabar, K. C. *et al, Anal. Chem.* 67:735-743 (1995), or through successively sonicating for 20 minutes in water, methanol, and acetone. Glass slides were functionalized in a 5-10% solution of MPMDMS in methanol for 1 hour. Copious rinsing with methanol and then water were proceeded to remove any unbound MPMDMS. Functionalized substrates were coated with a 12-nm colloidal Au solution for 90 or 95-s, depending on colloid concentration. Substrates were then rinsed with water and partially dried under an Ar stream. After exposure to LI Ag solution for 24 minutes (producing a 2-layer SERS-active substrate, *i.e.*, Ag-clad Au colloid), a non-continuous, 20-nm Ag film was thermally evaporated on the top of the substrate to produce the 3-layer Au-Ag-Ag substrate. Thermal evaporation was performed using an Edwards Auto 306 evaporation system. Metal deposition occurred at a pressure of 2×10^{-6} mbar and various deposition rates, with constant sample rotation to ensure even evaporation. All substrates were used immediately after evaporation.

Optical spectra were taken after each step above to monitor the surface quality. Film integrity was characterized by UV-visible absorbance spectroscopy, with quality control based on the shape and height of the Au plasmon peak. Suitable substrates were then used to form the 2-layer substrate as described above. The surface plasmon peak in the UV spectrum of the 2-layer substrate is shifted relative to the Au colloid alone, probably due to the optical property of coated Ag. In addition, the 2-layer substrate has a new shoulder around 620 nm caused by the particle coupling effect. After the discontinuous Ag

overlayer was evaporated over the 2-layer substrate (3-layer substrate), the shoulder became more pronounced as the result of stronger coupling from increased surface coverage.

Example 2

SERS Spectra of Various Substrates

Trans- 1,2-bis(4-pyridyl) ethylene (BPE) was obtained from Aldrich. BPE was recrystallized several times before use, and a stock 10-mM BPE solution was then prepared in a fresh H_2O:CH_3OH (9:1) solvent solution. Dilutions were made as needed to yield 1 mM, 0.1 mM, and 0.01 mM solutions in H_2O. These solutions were then drop-coated onto the various SERS substrates. SERS spectra were obtained on a Solution 633 micro-Raman spectrometer purchased from Detection Limit, Inc. Excitation was provided by a 20-mW, 632.8-nm HeNe laser. All spectra were taken with a 3-mm focal-length objective (~5 μm diameter in the spot size). Spectra were collected by a CCD detector with TE-cooling system around ~8.5°C. The system was operated and monitored by Labview software on a Monorail laptop (Monorail Corp.). SERS response was optimized by manually adjusting the height of the probe that was attached to a translation stage. Spectral analysis was handled with either Grams 32 or Igor Pro software packages.

Example 3

CE-SERS

A CE system was constructed similar to those previously described in Tracht *et al*, *Anal. Chem.* 66:2382-2389 (1994) and in Olefirowicz, T. M.& Ewing, A. G., *J. Chromatogr.* 499:713-719 (1990), each of which is incorporated herein by reference in their entirety. A 50-μm inner diameter (i.d.) fused silica capillary, 75 cm in total length and 65 cm in effective length for on-line UV detection, was used. Sample injection was accomplished via a hydrostatic method, as described in Landers, J. P. Handbook of capillary electrophoresis; CRC Press (1994), in Engelhardt *et al, Angew. Chem., Int. Ed. Engl.* 32:629-649 (1993), and in Weinberger, R. Practical capillary electrophoresis; Academic Press: Boston (1993) each of which is incorporated herein by reference in its entirety. Specifically, a 10-cm height difference between the two ends of the capillary was kept for 10 s with the injection end immersed in the sample vial (ca., 3×10^{-11} mole

analyte injection). The running buffer was 50 mM, pH 6.8 phosphate buffer. A Spellman CZE100OR power supply was used to apply a 15-kV potential on the capillary with the current kept at 40 μA. As a safety measure, the CE system was contained within a Plexiglas box secured with interlock; thus the power supply would automatically shut off when the lid was open. The capillary was activated and cleaned subsequently with 1 M NaOH, 18 M Ω H_2O, and 50-mM phosphate buffer at the beginning of every day. Before each injection, the capillary was again flushed with a fresh buffer solution. A linear variable-wavelength UV-visible detector was used for on-line detection in order to compare results with those acquired by SERS.

Surface Enhanced Raman Scattering: A Solution 633 Fiber-Optic microRaman Spectrometer with a remote probe was used for acquisition of Raman spectra. A He-Ne laser (632.8 nm) was used as the excitation source, and microscope objectives with focal lengths of 3 mm or 8 mm were used to collect spectra. The laser light focusing step was accomplished by adjusting the probe height manually. A light filter was used to adjust the output power from 0 - 20 mW. Spectra were collected by a TE-cooled CCD camera.

The interface between CE and SERS is presented in FIGURE 4. A 2-layer SERS susbtrate (Ag-clad Au colloid) was placed under the metallized outlet of the capillary. A translation stage, controlled by two 850-B actuators (Newport Corp.), was used to move the SERS substrate in the x and y axes at 0.8 mm/s. The analyte was deposited directly onto the substrate during separation while the substrate was moving in a continuous 'S' fashion.

For time course experiments (where integrated SERS intensity at a certain wavenumber range is plotted against elution time), the substrate was translated following eluant deposition to a position underneath the microRaman probe and the path of CE eluant deposition was retraced at the same speed for SERS data acquisition. Then, SERS intensities within the range of wavenumbers indicated in the text were acquired using an integration time of 1 s with a 1 s delay. For integrated SERS intensity measurements, a background for normalization was obtained by integrating the SERS intensity within a wavenumber range in which none of the analytes of interest displayed a Raman response.

Table 1. Comparison of Reproducibility for SERS Spectra of 10 mM BPE at Five Different Spots on the Same 3-Layer Substrate.

Peak Position (cm^{-1})	Mode[a]	Integrated Peak Area					Standard Deviation	Relative Average Deviation (%)
		Spot 1	Spot 2	Spot 3	Spot 4	Spot 5		
1010	Ring breathing	111,199	94,017	123,160	112,833	92,874	13,046	12
1200	C=C stretch	322,159	289,952	381,262	350,158	284,788	40,773	13
1610 & 1640	Ring stretch	749,191	705,067	936,888	844,896	706,120	100,613	13

Table 2. Comparison of Reproducibility for SERS Spectra of 10 mM BPE on Six, 3-Layer Substrates Prepared in Different Batches.

Peak Position (cm^{-1})	Mode[a]	Integrated Peak Area						Standard Deviation	Relative Average Deviation (%)
		Batch 1[b]	Batch 2[c]	Batch 3[d]	Batch 4[e]	Batch 5[f]	Batch 6[g]		
1010	Ring breathing	109,355	79,214	90,736	89,010	94,017	99,043	10,141	11
1200	C=C stretch	291,992	268,512	302,582	287,402	289,952	303,797	12,792	4
1610 & 1640	Ring stretch	609,668	554,222	678,806	624,567	705,067	774,308	77,973	12

Claims

What is claimed is:

1. A Surface Enhanced Raman Scattering (SERS)-active substrate comprising:
a solid support;
a submonolayer of microparticles attached to said solid support, each said microparticle comprising a colloidal particle of a first noble metal and an external coating of a second noble metal; and
a discontinuous film of said second noble metal, said discontinuous film positioned over said solid support and said microparticles.

2. The SERS-active substrate of claim 1, wherein said first noble metal is Au, and said second noble metal is Ag.

3. The SERS-active substrate of claim 2, wherein said discontinuous film is 10 nm – 30 nm in thickness.

4. A Surface Enhanced Raman Scattering (SERS)-active substrate comprising:
a solid support;
a submonolayer of microparticles attached to said solid support, each said microparticle comprising a colloidal Au particle coated with Ag; and
a discontinuous film of Ag, said discontinuous film positioned over said solid support and said microparticles.

5. The SERS-active substrate of claim 1, wherein said discontinuous film is evaporated over said solid support and said microparticles.

6. A method for forming a SERS-active substrate, the method comprising the steps of:
providing Au colloid particles,
providing a solid support capable of binding to said Au colloid particles;
contacting said Au colloid particles with said solid support under predetermined conditions to form a submonolayer of said Au colloid particles bound to said solid support;

contacting said Au colloid particles bound to said solid support with an Ag plating solution, whereby said Au colloid is coated with Ag; and

evaporating a discontinuous film of Ag over said solid support.

7. An instrument for depositing analytes resolved by capillary electrophoresis (CE) onto a Surface Enhanced Raman Scattering (SERS)-active substrate, said analytes contained in eluant, the instrument comprising:

a CE unit comprising a capillary with an outlet from which eluant is discharged during CE;

a SERS substrate, said SERS substrate positioned adjacent to said outlet such that eluant is deposited onto said SERS substrate during CE, said SERS substrate operationally associated with translation means capable of moving said SERS substrate in a defined pattern;

wherein movement of said SERS substrate in said defined pattern during CE leads to the formation of a linear eluant trail in which different analytes are deposited at different positions along said eluant trail.

8. A method for Raman-spectroscopic analysis of analytes resolved by capillary electrophoresis (CE), said analytes contained in eluant, comprising:

providing a CE unit comprising a capillary with an outlet from which eluant is discharged during CE; whereby different analytes are discharged in eluant at different times;

providing a Surface Enhanced Raman Scattering (SERS)-active substrate, said SERS-active substrate positioned adjacent to said outlet such that eluant is deposited onto said SERS-active substrate during CE, said SERS substrate operationally associated with translation means capable of moving said SERS substrate in a defined pattern;

performing CE and simultaneously moving said SERS substrate in said defined pattern, wherein a linear trail of eluant is deposited on said SERS substrate, and wherein different analytes are deposited in eluant at different positions along said eluant trail; and

examining said eluant trail by Raman spectroscopy.

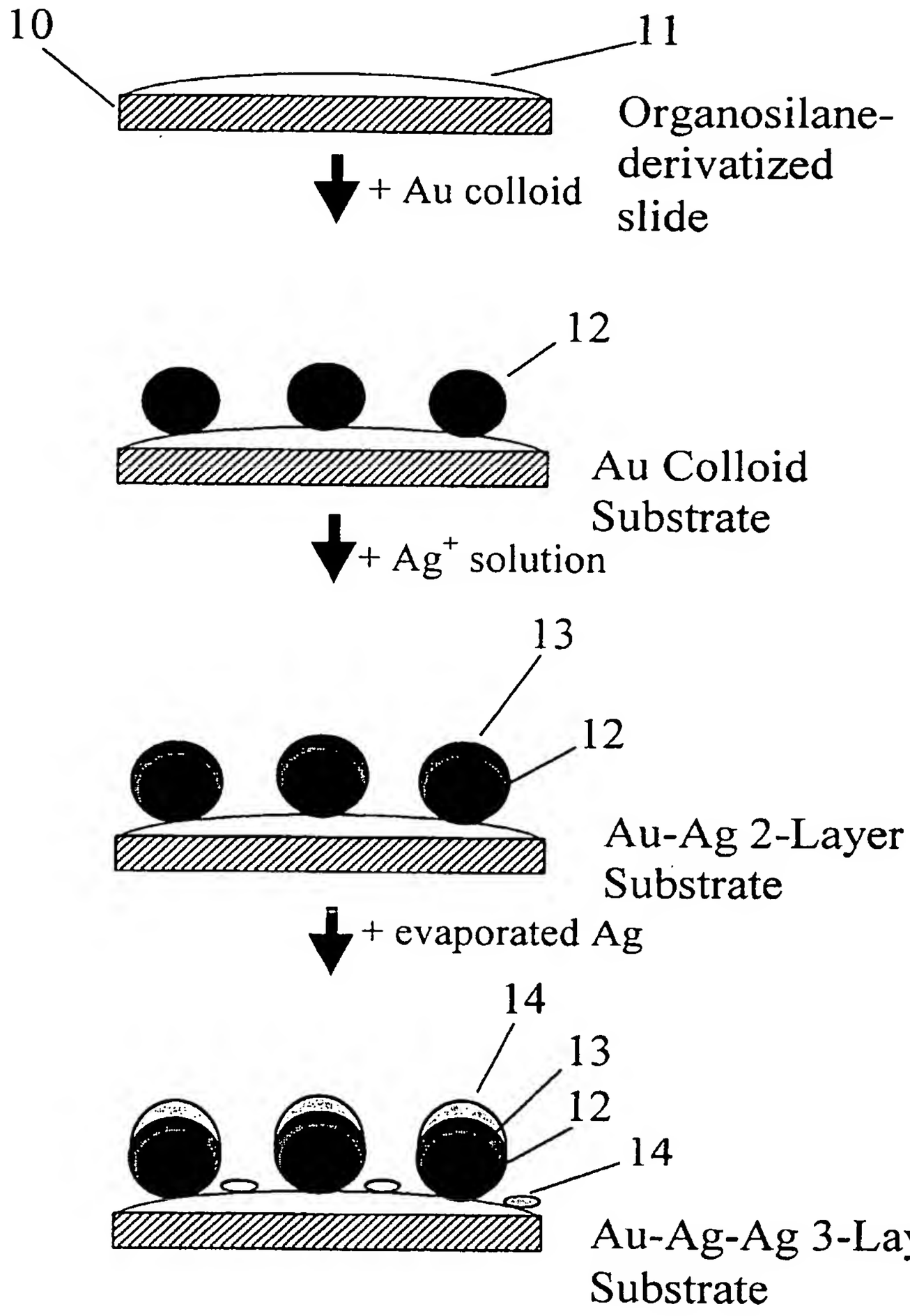
10
11
Organosilane-derivatized slide
+ Au colloid
12
Au Colloid Substrate
+ Ag+ solution
13
12
Au-Ag 2-Layer Substrate
+ evaporated Ag
14
13
12
14
Au-Ag-Ag 3-Layer Substrate

Fig. 1

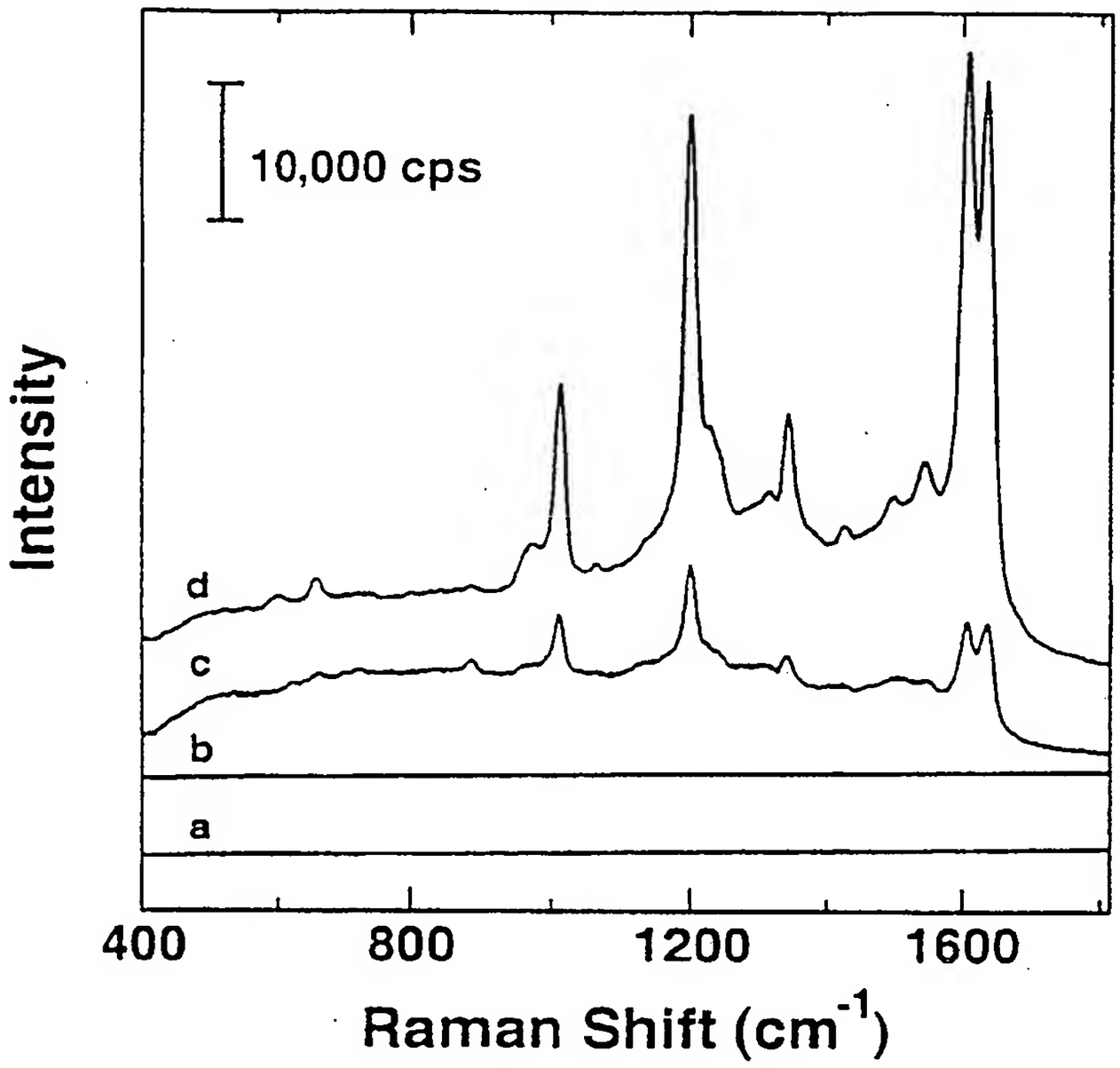

Fig. 2

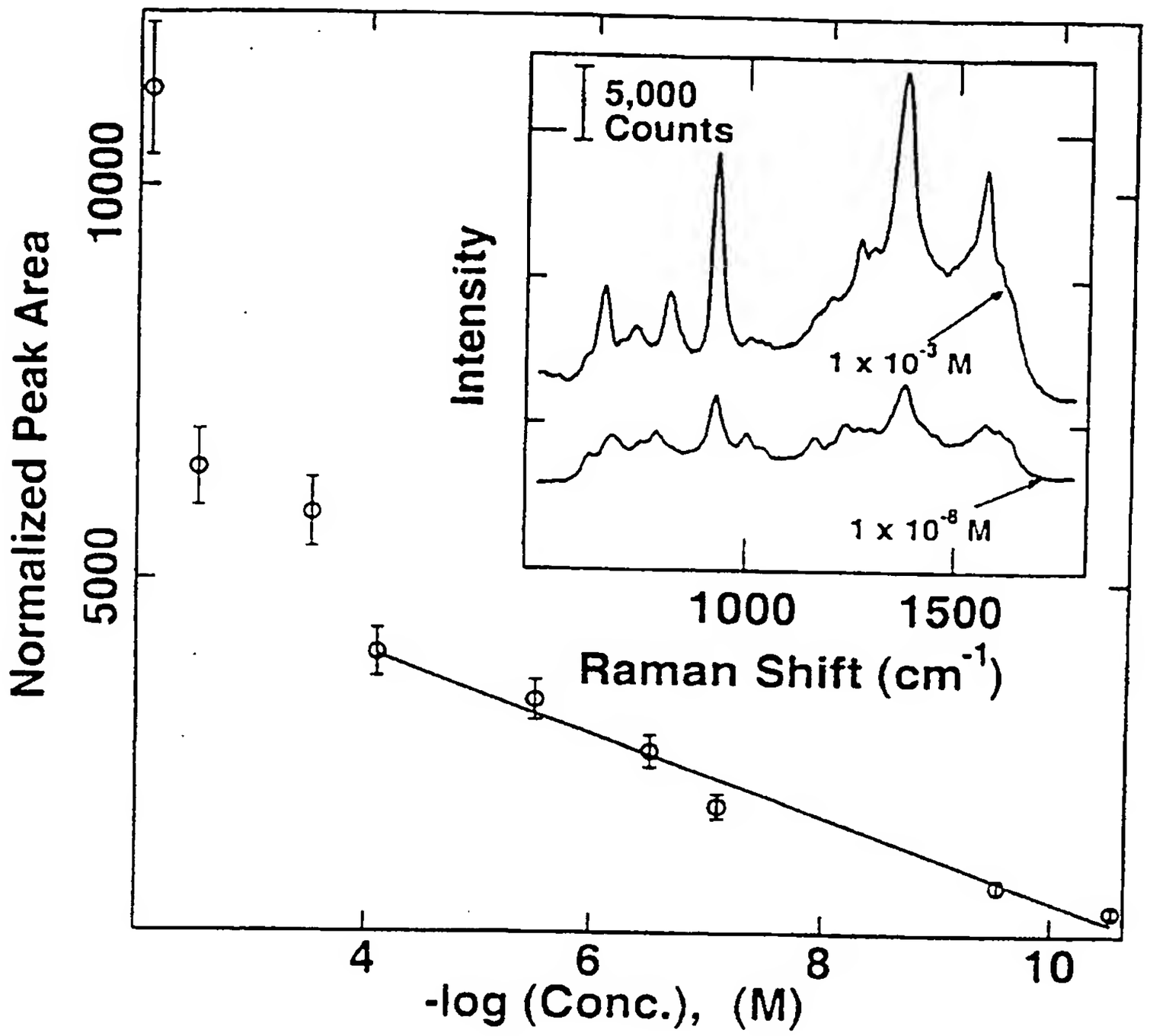
Normalized Peak Area
10000
5000
4
6
8
10
-log (Conc.), (M)
Intensity
5,000 Counts
1 x 10⁻³ M
1 x 10⁻⁸ M
1000
1500
Raman Shift (cm⁻¹)

Fig. 3

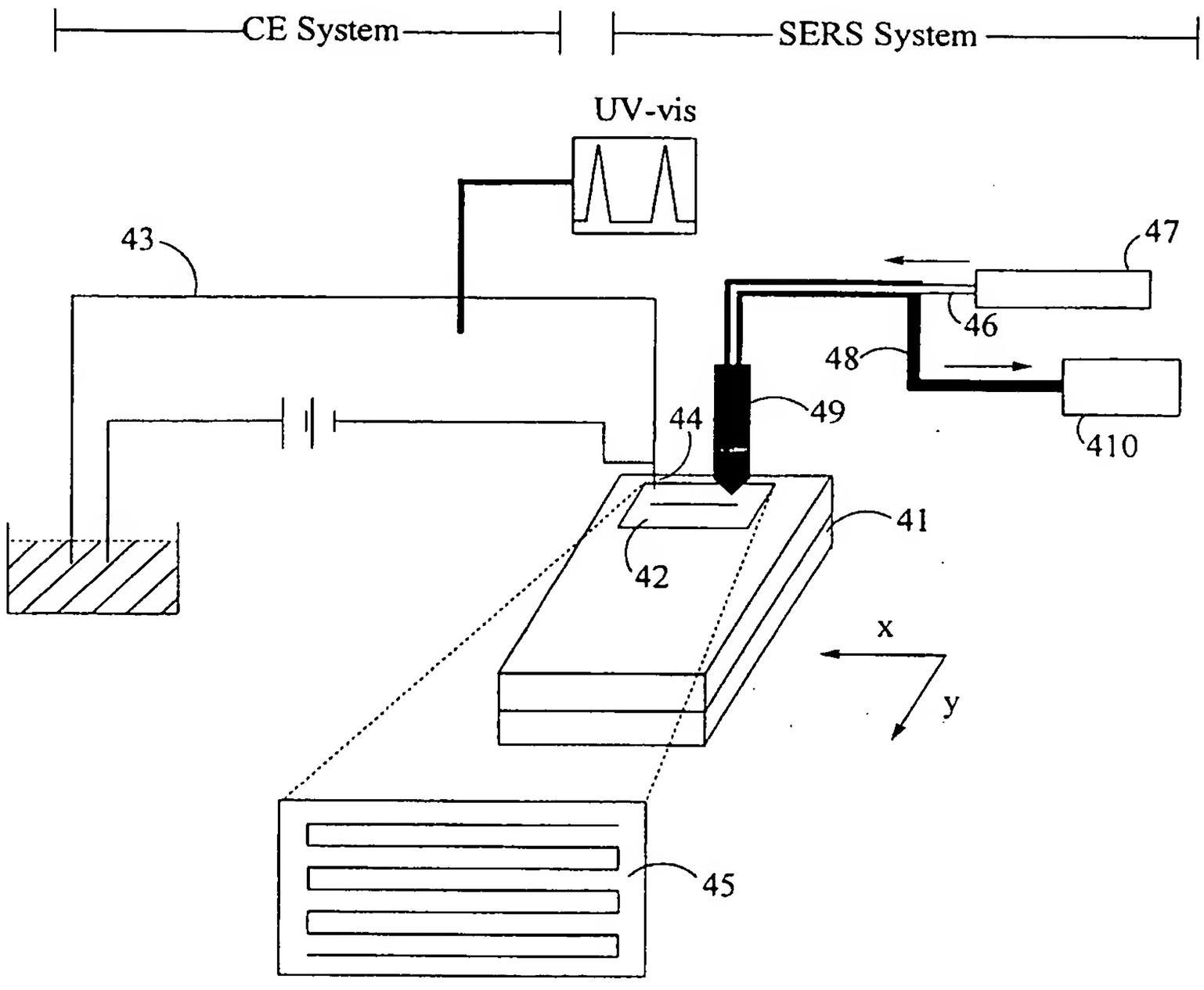

Fig. 4

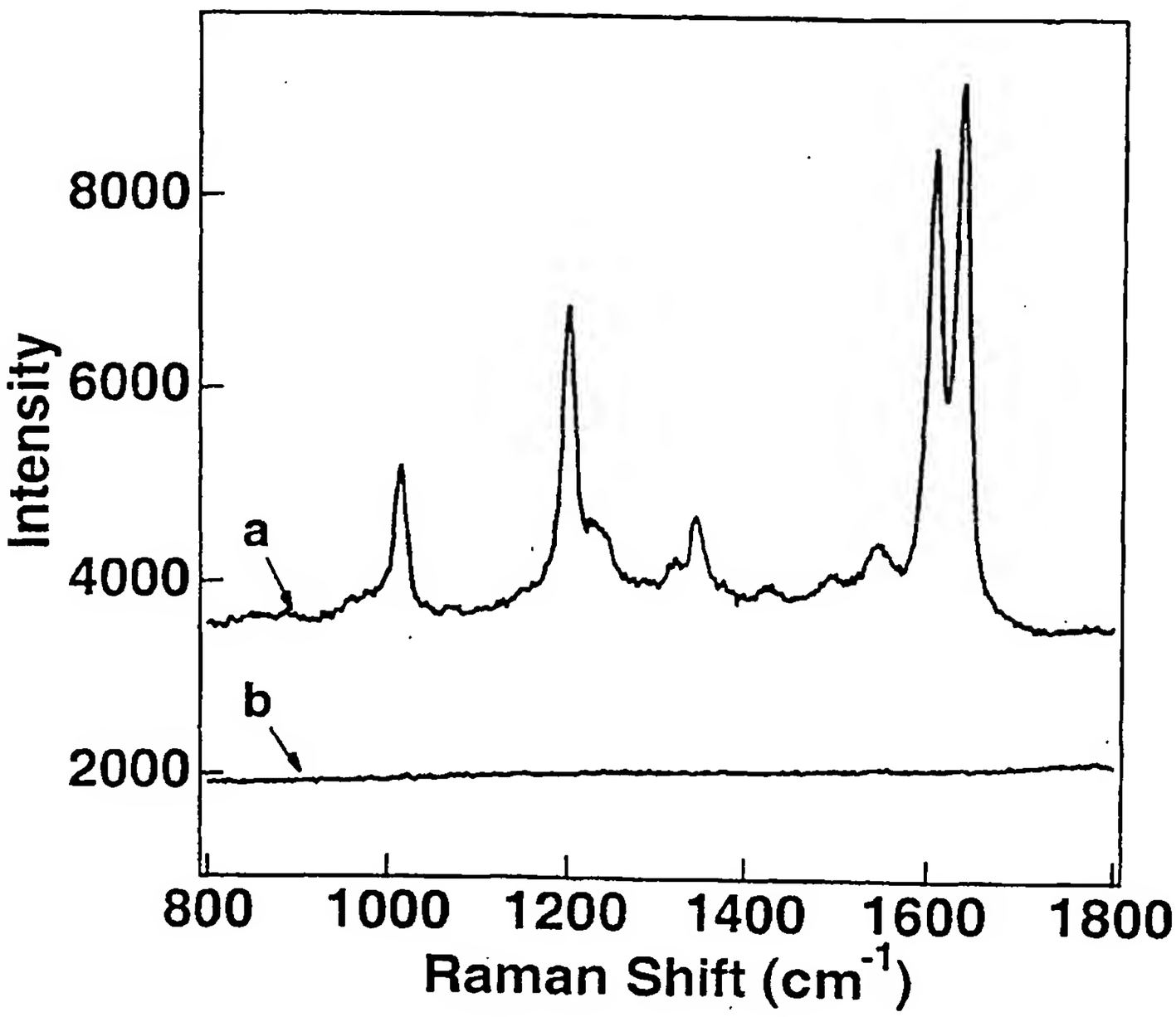
8000
6000
4000
2000
Intensity
a
b
800
1000
1200
1400
1600
1800
Raman Shift (cm-1)

Fig. 5

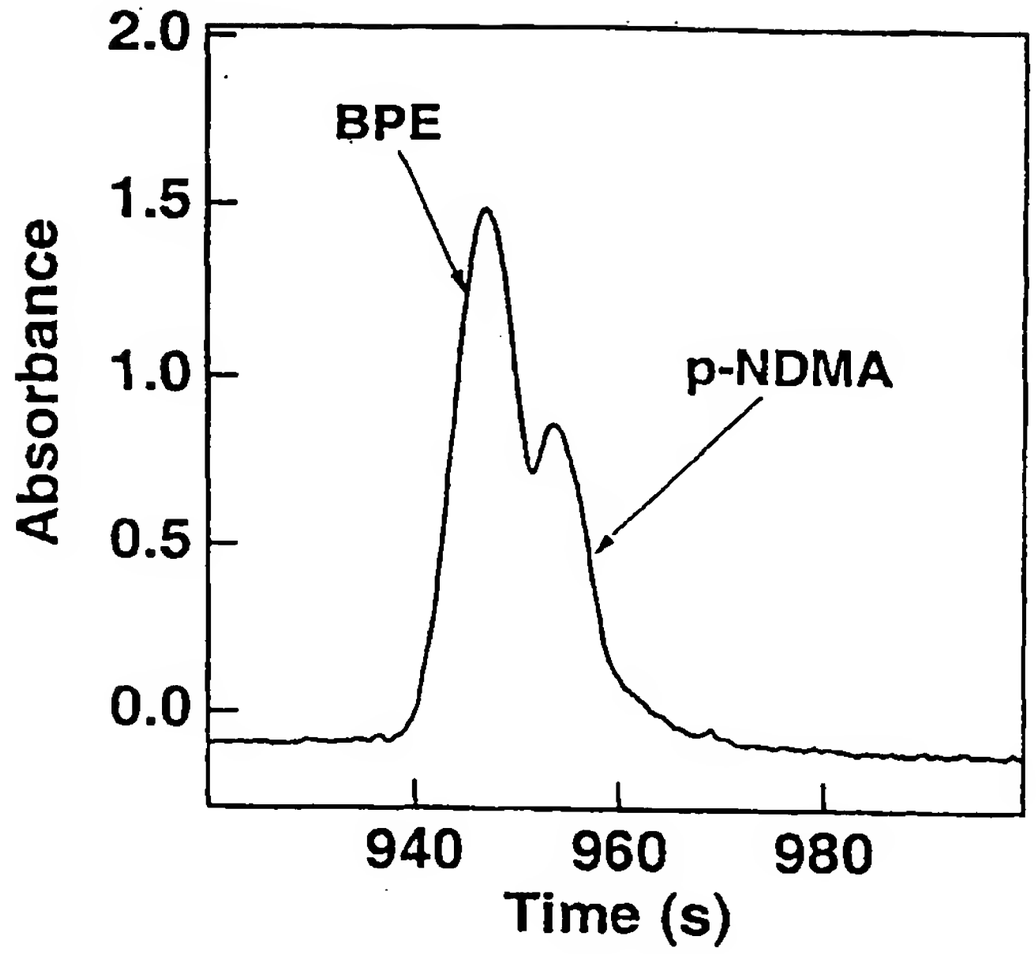
2.0
1.5
1.0
0.5
0.0
Absorbance
BPE
p-NDMA
940
960
980
Time (s)

Fig. 6A

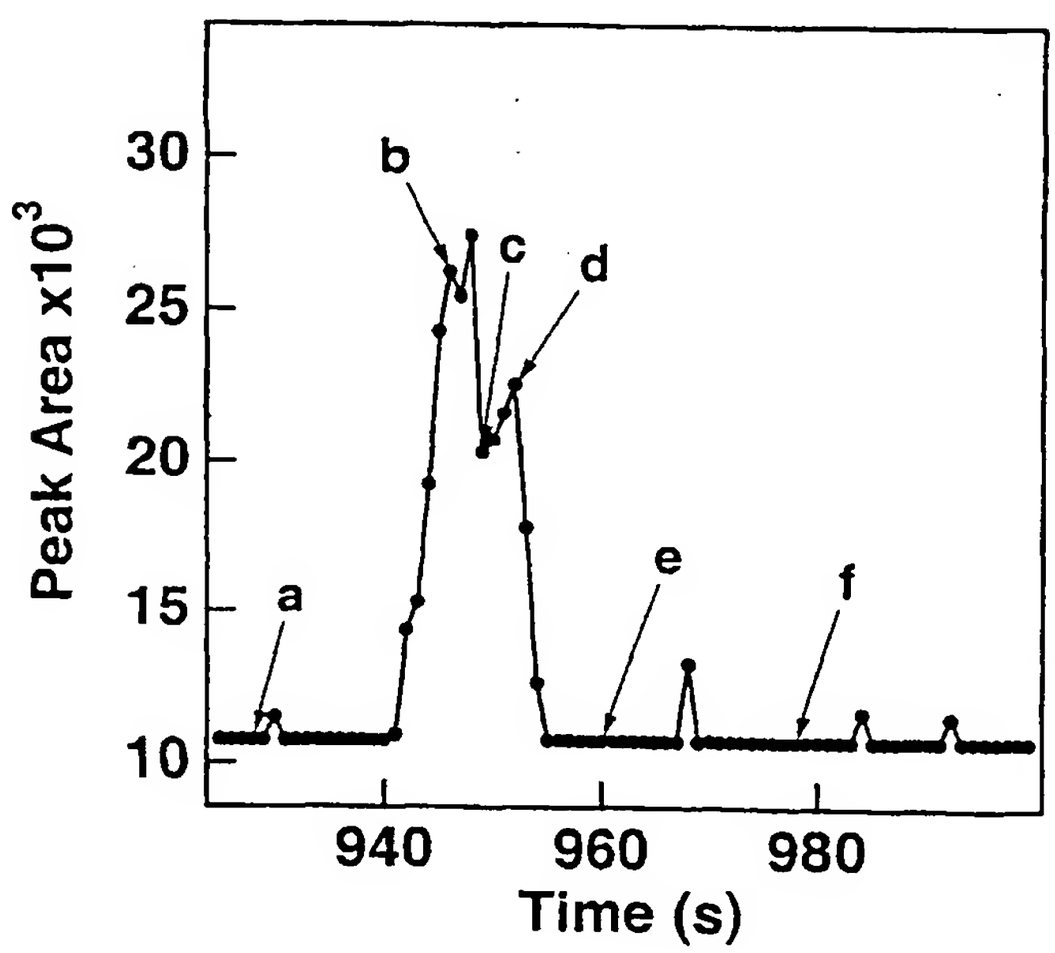
30
25
20
15
10
Peak Area x10^3
a
b
c
d
e
f
940
960
980
Time (s)

Fig. 6B

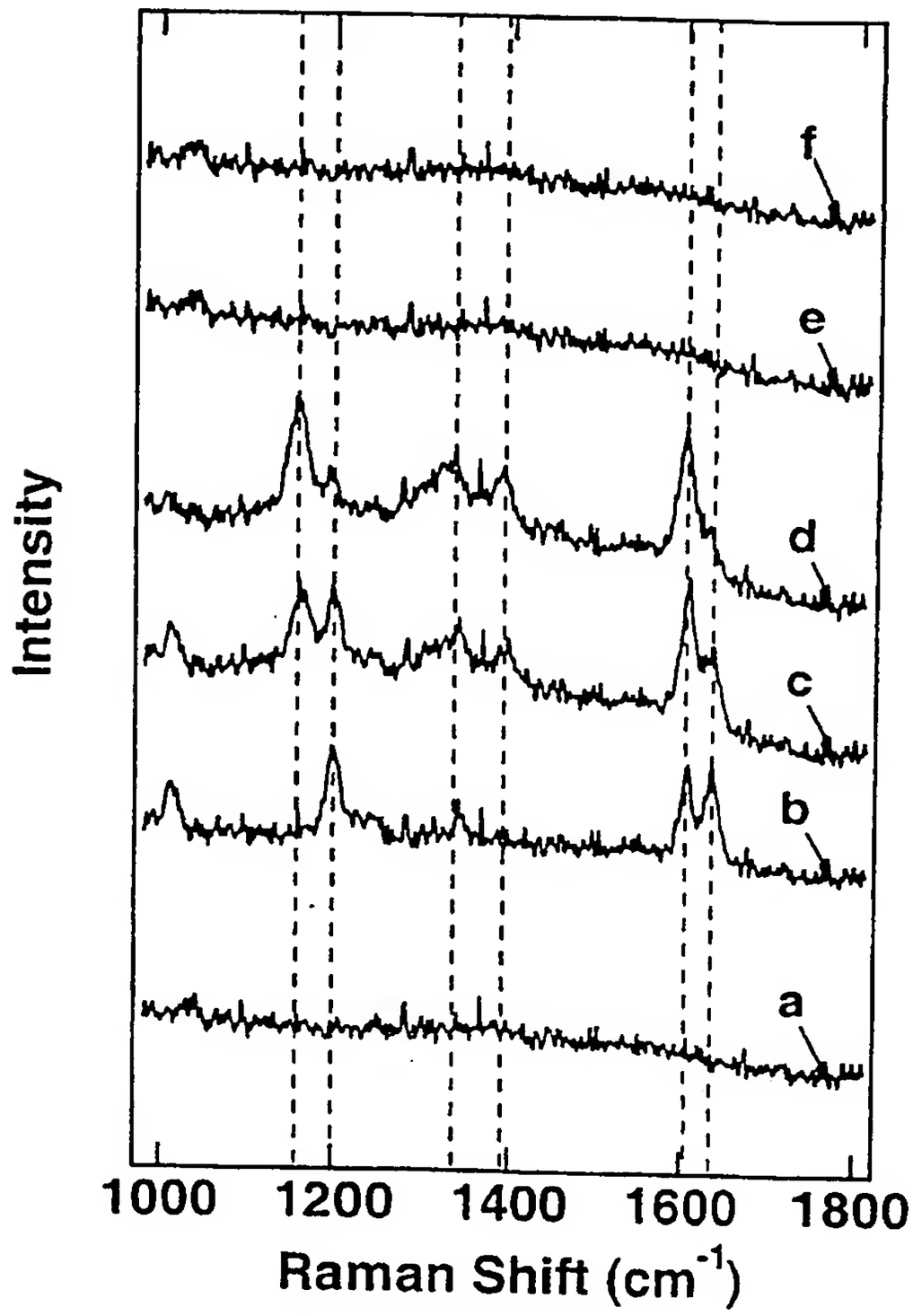

Fig. 7

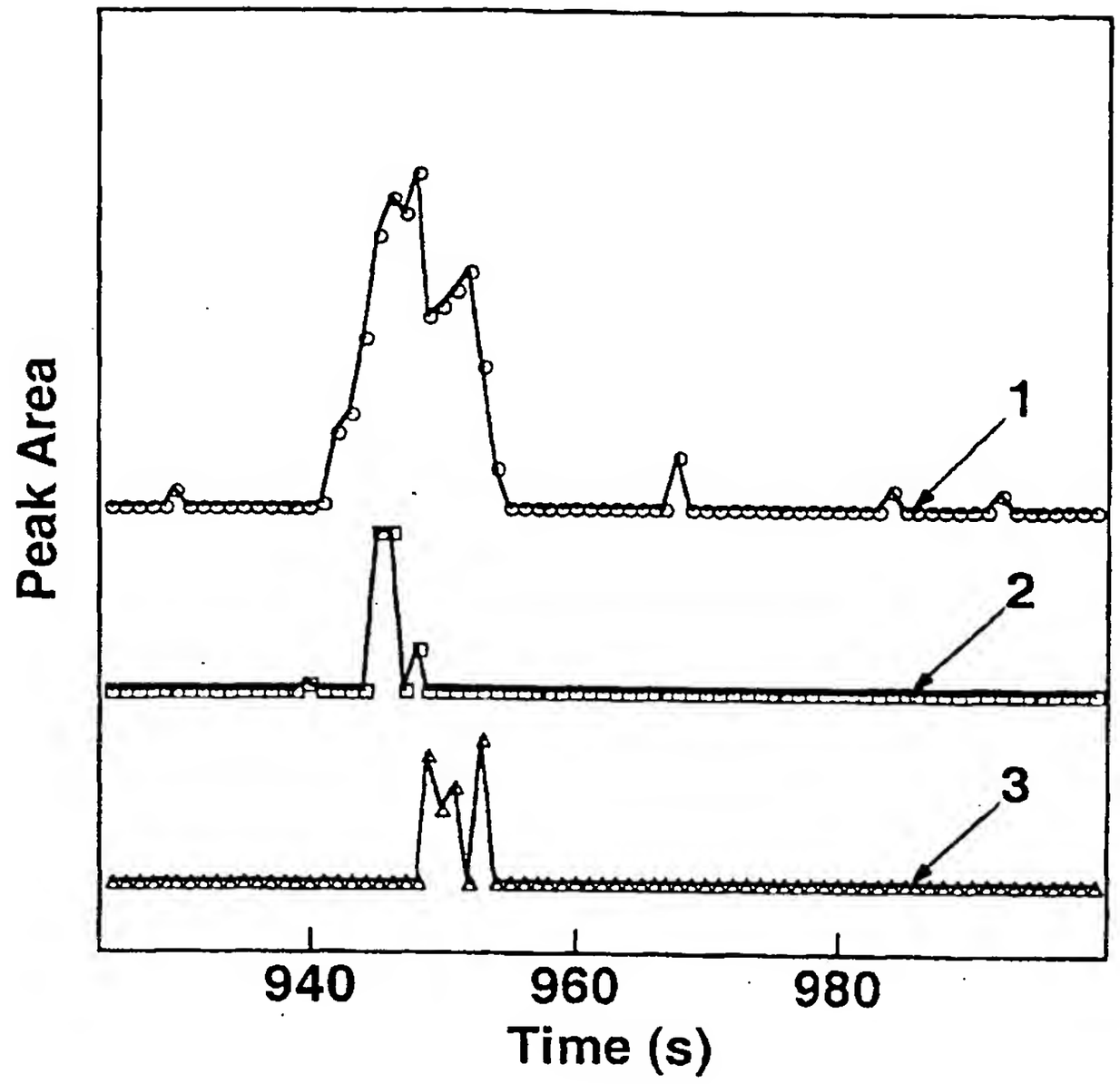

Fig. 8

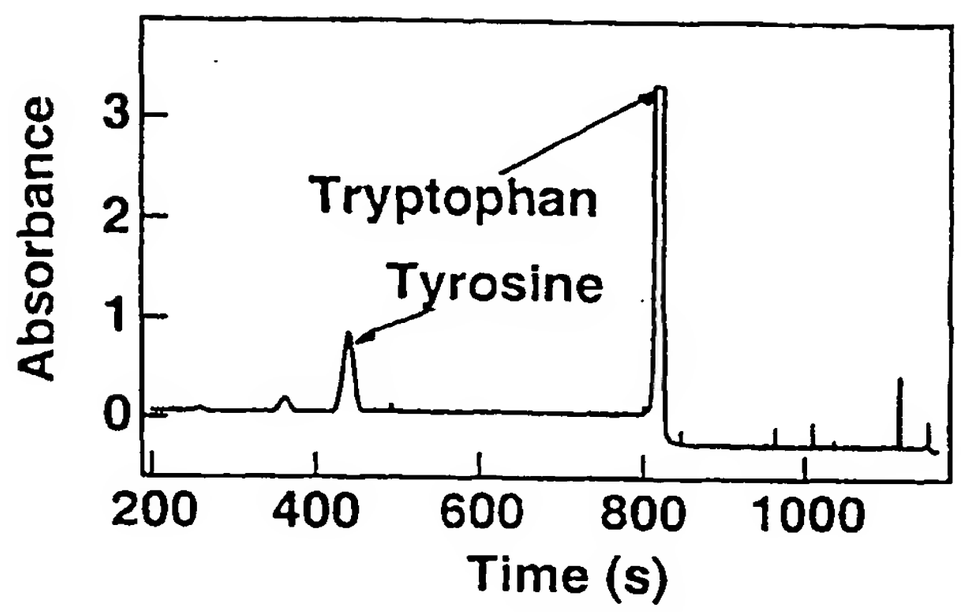

Fig. 9A

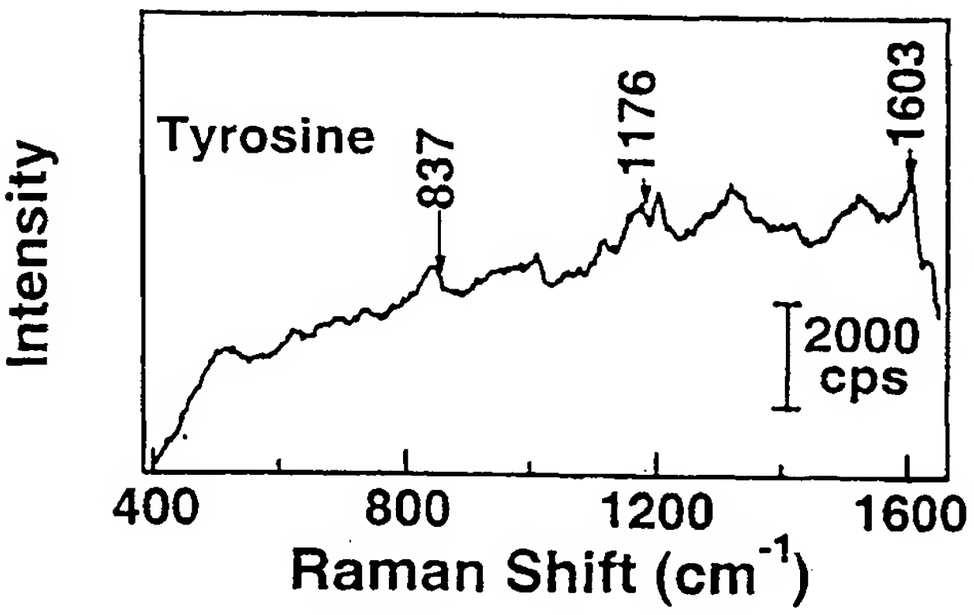

Fig. 9B

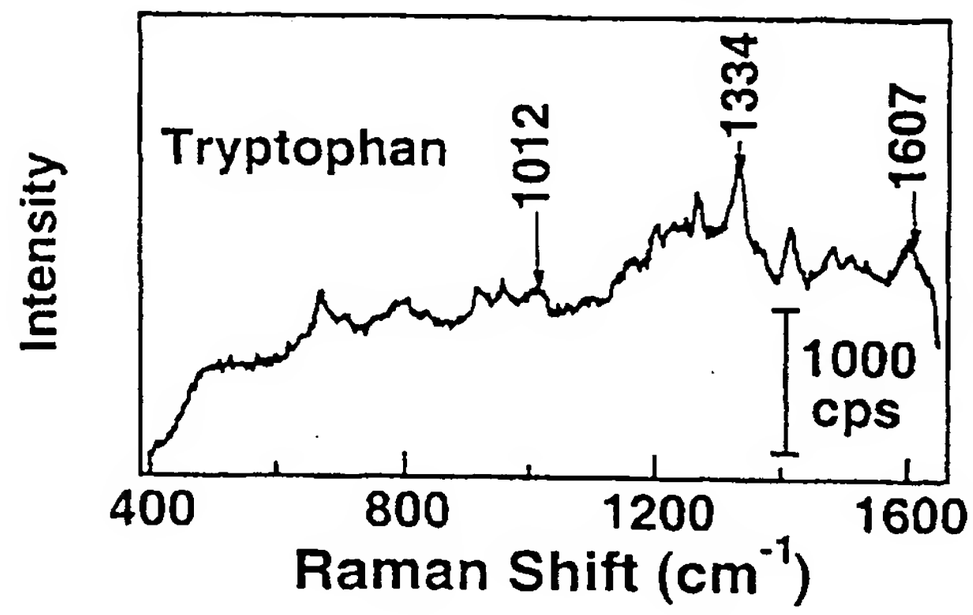

Fig. 9

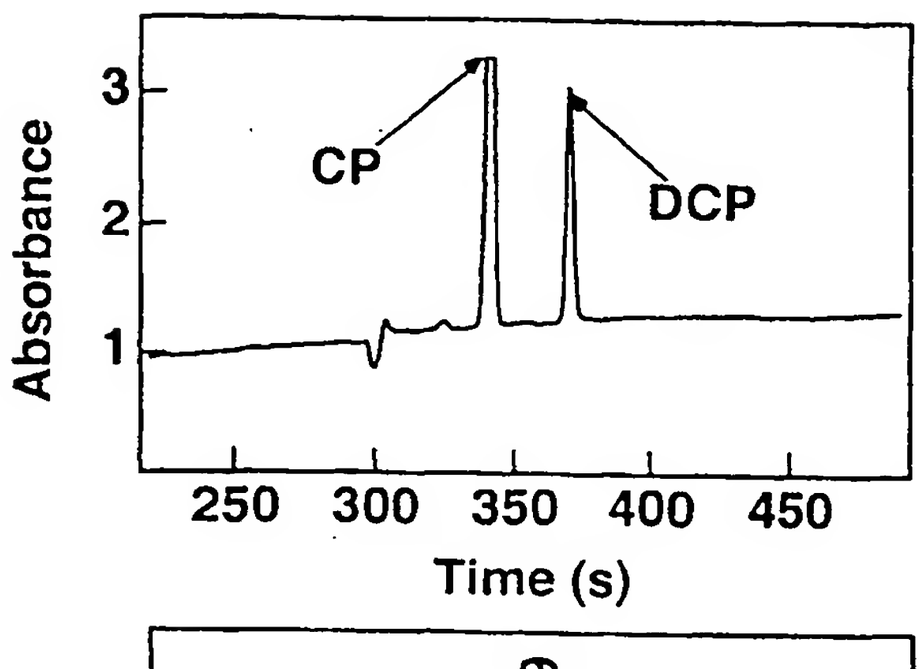

Fig. 10A

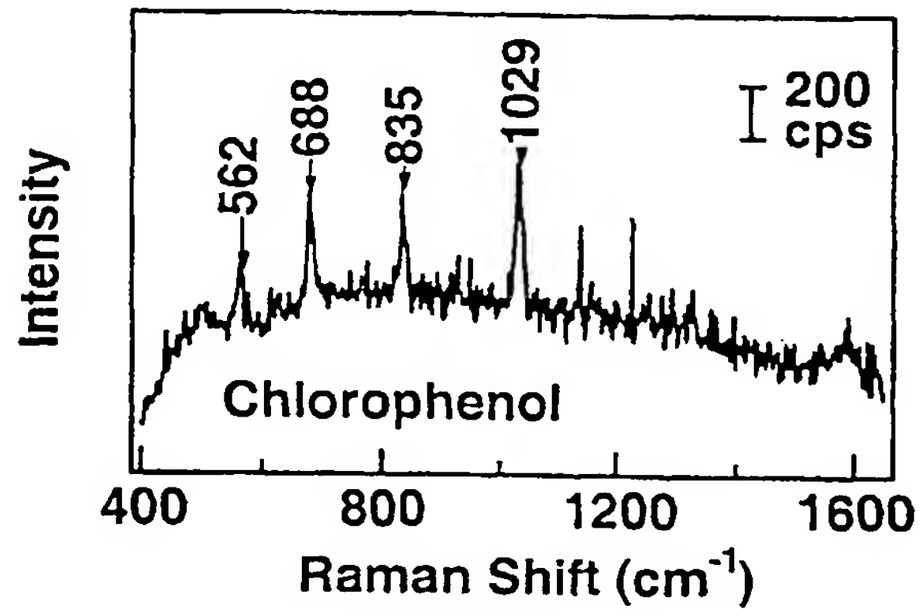

Fig. 10B

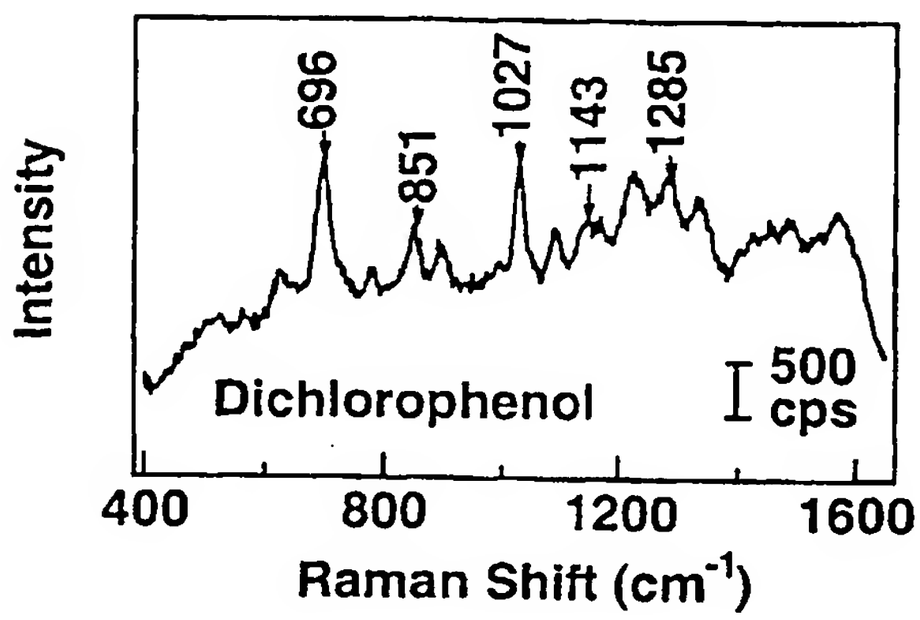

Fig. 10C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27667

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G01N 21/65
US CL :422/82.05; 436/164; 356/301

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/82.05, 82.09; 436/164, 525; 356/301; 250/458.1, 459.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPTO APS EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,E	US 6,149,868 A (NATAN et al) 21 November 2000 (21.11.2000), see entire patent.	1-8
A	US 5,828,450 A (DOU et al) 27 October 1998 (27.10.1998), see entire patent.	1-8
A	US 5,266,498 A (TARCHA et al) 30 November 1993 (30.11.1993), see entire patent.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 DECEMBER 2000	09 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer FRANK FONT Telephone No. (703) 308-0956

Form PCT/ISA/210 (second sheet) (July 1998)★